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THE EFFECT OF CERTAIN AROMATIC COMPOUNDS ON ENZYMES INVOLVED IN--ETC(U)

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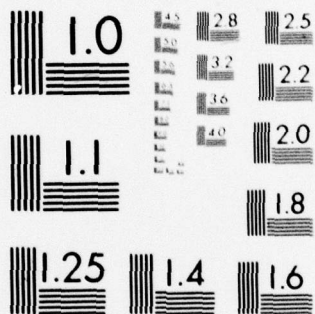
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THE EFFECT OF CERTAIN AROMATIC COMPOUNDS ON
ENZYMES INVOLVED IN HEME SYNTHESIS,

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Benzene, toluene and some of their nitro, amino and chloro derivatives often become environmental pollutants during the process of munitions manufacture. The full assessment of the ecological impact made by these compounds inadvertently released into the environment has not been made.

Because of the quantities manufactured, we focused much of our attention on the trinitrotoluenes and dinitrotoluenes. Both trinitrotoluene and dinitrotoluene are found in "pink water," the water soluble effluent from factories making 2,4,6-trinitrotoluene. The 2,4,6-trinitrotoluene is the military high explosive produced in greatest quantity (1-2). This study was undertaken because benzene, trinitrotoluene and several other substituted benzenes and toluenes have been implicated as causative agents for both aplastic anemia (3-5) and leukemia (6-8). Benzene and, in some instances, toluene and xylene, have been shown to inhibit various steps in globin and heme synthesis in in vitro systems (9-10). We have utilized this property to study structure-activity relationships of benzene, toluene and many of their derivatives on two enzymes involved in heme synthesis. The enzymes are delta-aminolevulinic acid synthetase (ALAS) and heme synthetase (HS).

ALAS and HS were chosen for initial studies because they represent the starting and final enzymes in the synthesis of heme. ALAS is the first and presumably rate limiting enzyme in heme synthesis. Delta-aminolevulinic acid, (ALA), is the first stable product formed in the biosynthesis of heme. ALA is synthesized by the action

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of ALAS on glycine and succinyl Coenzyme-A with a requirement for pyridoxal-5'-phosphate as a cofactor. Schulman and Richert (11), and Lascelles (12) were the first to demonstrate that pyridoxal-5'-phosphate was a required cofactor for the production of ALA. Observations within our laboratory confirm these earlier findings. In addition to its position in the scheme of the biosynthesis of heme, its short half-life of 1-2 hours makes ALAS ideally suitable to play a regulatory role in heme synthesis.

HS, (ferrochelatase or ferrolyase), E.C.4.99.1.1, is the final enzyme in the production of heme. It catalyzes the insertion of ferrous but not ferric iron into the protoporphyrin-IX ring to produce heme. The incorporation of ferrous iron into the porphyrin ring may occur non-enzymatically under certain mild conditions (13-14); however, the conditions under which we evaluated this system, only the enzymatic incorporation of iron occurred. Some of these conditions include:

1. Maintenance of anaerobic conditions and iron in the ferrous state.
2. A pH optimum near 8; we analyzed at pH 7.5.
3. De-esterification of the porphyrin ester into the corresponding free acid. (15)

Meso- and deuteroporphyrins also allow the incorporation of ferrous iron into their ring systems. Our observations confirmed those of Bonkowsky's (16) in that of the three prophyrin isomers, proto-, meso-, and deuterio-, the meso- was the most active in the uptake of ferrous iron.

MATERIALS AND METHODS

The aromatic compounds studied (Figure 1) came from both commercial and "discreet" sources. Many of the compounds tested are available from the Aldrich Chemical Company, Milwaukee, Wisconsin 53233. We tested for impurities by subjecting each compound to thin-layer chromatography using two different solvent systems, benzene-ethyl acetate 1:4, and acetonitrile. Each compound tested showed only a single spot on migration on pre-coated silica gel plates (E.M. Laboratories, 500 Executive Blvd., Elmsford, N.Y. 10523). De-ionized water of at least 7 megohm resistance was used in the preparation of all aqueous reagents (17). All other reagents and solvents were ACS grade or better.

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Male rats, Walter Reed strain, 275-400g, were fasted overnight but given water ad libitum. The animals were sacrificed by asphyxiation in a carbon dioxide saturated atmosphere. The livers were quickly excised, blotted with gauze, weighed and placed in ice-cold 0.25 mol/l sucrose - .05 mol/l Tris buffer, pH 7.5, containing 1% Tween-20 (16). All subsequent operations of the tissue prior to incubation at 37°C were carried out in the cold. The tissue samples were homogenized and sonicated to assure disruption of the mitochondrial membrane. After sonication, the volumes were adjusted to provide a 5% w/v suspension of tissue homogenate in the buffer.

The determination for ALAS activity was performed by a modification of the method of Ebert, et al (18). The difference was that we used labeled alpha-ketoglutaric acid instead of labeled succinyl Co-enzyme A. HS was analyzed by a modification of the technique of Bonkowsky, et al (16). The details of this modification are in preparation for publication elsewhere.

Initially, we evaluated some of these compounds at 0.2 mol/l, which was the concentration of the aromatic with respect to the reaction mixture. Recognizing that at higher concentrations, some effect would be manifested, we focused our attention on concentrations which might be reasonably expected to become physiologically achievable. We extensively studied the effects in the 10^{-3} to 10^{-6} mol/l concentration range.

RESULTS

Figure 1 shows the structures of the compounds studied, most of which inhibited ALAS and enhanced HS activities. It is recognized that in mammalian adults, heme acts as an inhibitor or by means of a heme-controlled repressor, controls the additional production of heme by limiting the amount of ALA produced. We have not ascertained through our experiments whether or not the decrease in ALAS activity under the influence of the chemical is due to the presence of the chemical alone, additionally produced heme alone, or a combination of these two factors.

Table 1 gives the relative sensitivity of these enzymes toward the compound studied. The strata of sensitivity to ALAS are the dinitrobenzenes>trinitrotoluenes>dinitrotoluenes>amino-dinitrotoluenes>chlorotoluenes>benzene. For HS, the strata of sensitivity are the dinitrobenzenes>dinitrotoluenes>amino-dinitrotoluenes>trinitrotoluenes>nitrobenzene. As a class of compounds, the monochlorotoluenes had little effect on the enzyme systems. The addition of one nitro group to the benzene ring had little effect on ALAS

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and HS activities. The addition of a second nitro group had a marked effect on the activities of both enzymes, especially at 10^{-3} mol/l. At this concentration, the dinitrobenzenes gave an average increase of 48% on HS activity. This effect could be demonstrated at a concentration as low as 10^{-5} mol/l in which one gets an average enhancement of 35% for this enzyme. Conversely, at 10^{-3} mol/l, the activity of ALAS was decreased an average of 44% and at 10^{-5} mol/l, the average was essentially the same as the control value.

The enhancement/inhibition effect of placing the second nitro group on the aromatic ring is also evident with 4-nitrotoluene and the dinitrotoluenes. Toluene and 4-nitrotoluene have little effect on ALAS and HS activities; however, when the second nitro group is introduced to the ring, there is an average decrease of 37% in ALAS activity and an average increase of 26% in HS activity.

One may note in Table 1 that on replacing the nitro group by an amino group, there is a tendency to further inhibit ALAS and enhance HS activities. The reverse trend occurs in the tri-substituted toluenes in which one nitro group has been replaced by an amino group.

Figure 2 shows the comparison of the activities of the dinitrobenzenes on ALAS and HS activities. It may be noted that in concentrations greater than 10^{-5} mol/l, as HS activity increases, ALAS activity decreases. The differences between ALAS and HS activities at 10^{-3} mol/l cannot be solely attributed to aqueous solubilities of the isomers because their rank in water solubilities is m>o>p-isomers respectively, whereas their effect on the enzymes is p>o>m. The effect may be due to steric specificity and/or spatial arrangements of the molecules involved.

Figure 3 shows the comparison of the effect of the dinitrotoluenes on the enzymes studied. At the same concentrations with respect to the dinitrobenzenes, the effect is not as pronounced which suggests that there may be some interaction between the methyl and nitro groups which offsets the enhancement/inhibition effects. A similar trend is noted with the trinitrotoluenes and the amino-dinitrotoluenes which is shown in Figure 4. The addition of an amino group to the aromatic ring appears to lessen the decrease in ALAS activity.

The normal values obtained for rat liver ALAS were 1-3 nmol/g protein/0.5 hr and 8-14 μ mol/g protein/0.5 hr for HS.

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DISCUSSION

We have utilized the sensitivity of two enzymes involved in heme synthesis to selected organic compounds as a means of assessing pharmacological effects of aromatics of interest on the activity of these enzymes. ALAS and HS were chosen for these studies because they represent the initial and final enzymes in the biosynthetic pathway of heme. In addition, both have regulatory roles in the production of heme.

Several investigators have explored the effect of benzene on heme synthesis. It has been shown by Forte, et al (10) and Wildman, et al (9) that benzene, at a final concentration of 0.113 mol/l, inhibits rabbit reticulocyte heme synthesis in vitro at the ALAS step. Lee, et al (19) have shown that a single subcutaneous dose of benzene in mice decreases the incorporation of ^{59}Fe into erythrocytes, 440 mg/kg body weight produced a 27% and a dosage of 2200 mg/kg producing a 50% inhibition. A smaller dose, 88 mg/kg had no effect on the incorporation of ^{59}Fe and no effect on the hematocrit.

Other groups have examined structure-activity relationships of various classes of compounds with respect to their effect on ALAS and other related enzymes. Marks, et al (20), investigated the porphyrin-inducing activity of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethyl pyridine, a compound known to induce porphyria by enhancing the synthesis of ALAS, and several analogues of it and the corresponding pyridines. They determined the porphyria-inducing activity of these compounds by feeding them to guinea pigs and estimating the amount of ALA and porphobilinogen excreted in the urine. Poland and Kende (21) investigated a number of halogenated di-benzo-p-dioxins for their ability to stimulate two hepatic enzymes: (1) ALAS and (2) aryl-hydrocarbon hydroxylase, a cytochrome P-450 mediated microsomal mono-oxygenase. The potency of the halogenated di-benzo-p-dioxins to induce ALAS and aryl-hydrocarbon hydroxylase corresponded precisely with their lethal, teratogenic and acnegenic potency, to the extent that toxicological data were available.

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In summary, we have used techniques of measuring two enzymes involved in heme synthesis to study in vitro effects of certain organic chemicals on these enzymes as a means of determining structure activity relationships for in vivo predictions. We have attempted to use physiologically attainable concentrations of the chemicals in question and have demonstrated that an effect can be registered routinely at concentrations as low as 10^{-5} mol/l and in some cases as low as 10^{-6} mol/l.

10 to the minus 6th power

10 to the minus 5th power

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The utility of these techniques lie in the versatility of measuring the levels of these enzymes not only in liver hemogenates, but also in bone marrow aspirates and reticulocyte rich blood. Bone marrow or whole blood measurements may obviate the need for surgical procedures when monitoring the pharmacological effect of the compounds of interest.

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Table 1. The effect of the compounds investigated on the two enzyme systems studied.

Concentration of Aromatic Test	Percentages of Control							
	10 ⁻³ mol/l		10 ⁻⁴ mol/l		10 ⁻⁵ mol/l		10 ⁻⁶ mol/l	
	ALAS	HS	ALAS	HS	ALAS	HS	ALAS	HS
Aromatic								
Benzene	95	86	104	91	107	94	107	89
Toluene	96	92	104	86	93	91	100	89
Nitrobenzene	86	111	108	104	110	100	81	105
o-Dinitrobenzene	43	143	84	148	104	146	105	105
m-Dinitrobenzene	83	149	94	118	118	119	100	104
p-Dinitrobenzene	41	152	50	180	81	127	93	112
4-Nitrotoluene	84	93	95	99	96	107	96	113
2,3-Dinitrotoluene	55	130	81	103	97	85	104	99
2,4-Dinitrotoluene	92	87	100	80	102	92	99	96
2,5-Dinitrotoluene	43	139	91	102	89	82	90	66
2,6-Dinitrotoluene	77	117	87	96	100	89	97	84
3,4-Dinitrotoluene	56	131	86	119	97	87	99	94
2,3,4-Trinitrotoluene	26	104	69	119	101	96	99	100
2,4,6-Trinitrotoluene	86	150	93	136	103	117	105	119
4-Amino- 2,6-Dinitrotoluene	82	137	87	126	93	110	89	109
4-Amino- 3,5-Dinitrotoluene	89	152	94	140	107	113	107	105
2-Amino- 4-Nitrotoluene	89	97	88	111	87	118	94	103
o-Chlorotoluene	87	101	98	118	99	108	94	111
m-Chlorotoluene	97	107	99	110	101	104	103	104
p-Chlorotoluene	97	108	100	108	102	106	103	104
1-Chloro- 2-Nitrobenzene	102	115	88	113	95	109	99	110
Hexachlorobenzene	117	133	94	131	99	116	104	113
Aniline	96	108	91	121	94	104	98	116

STRUCTURES OF AROMATICS

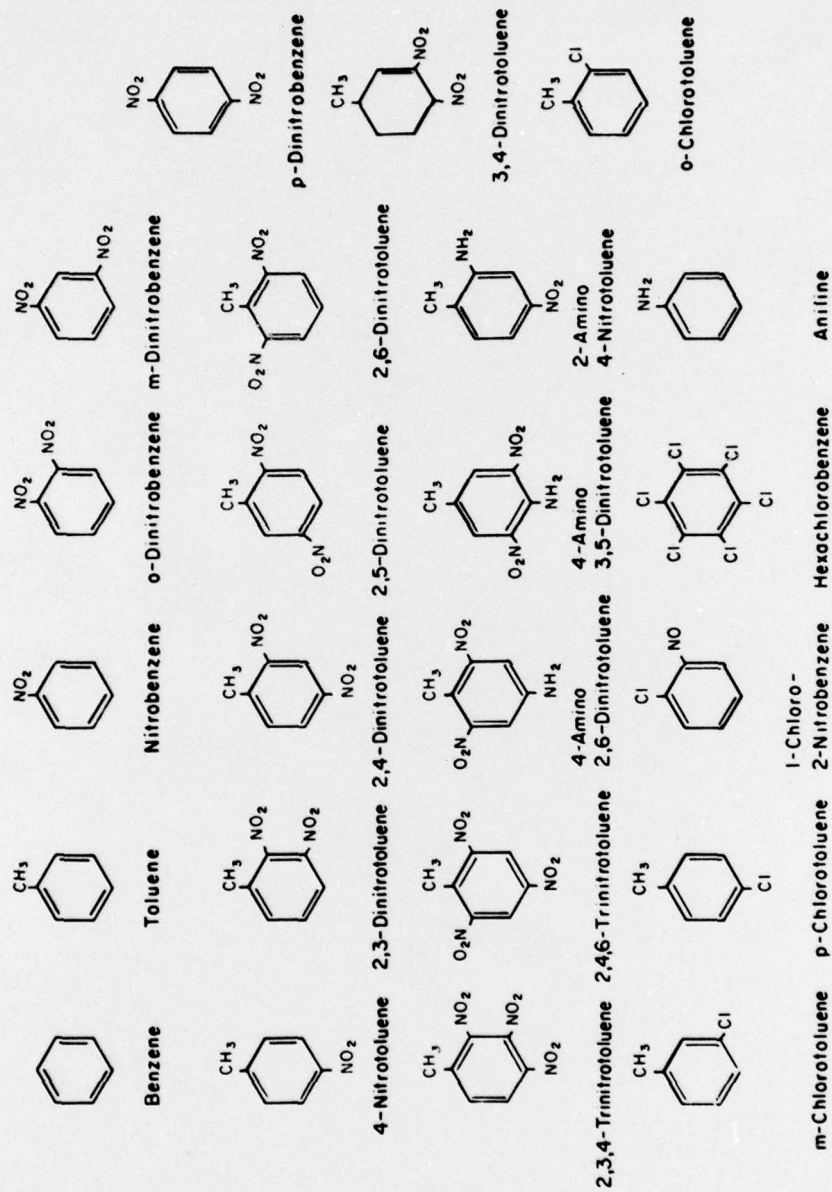


Fig. 1. Structures of the compounds under investigation.

THE DINITROBENZENES

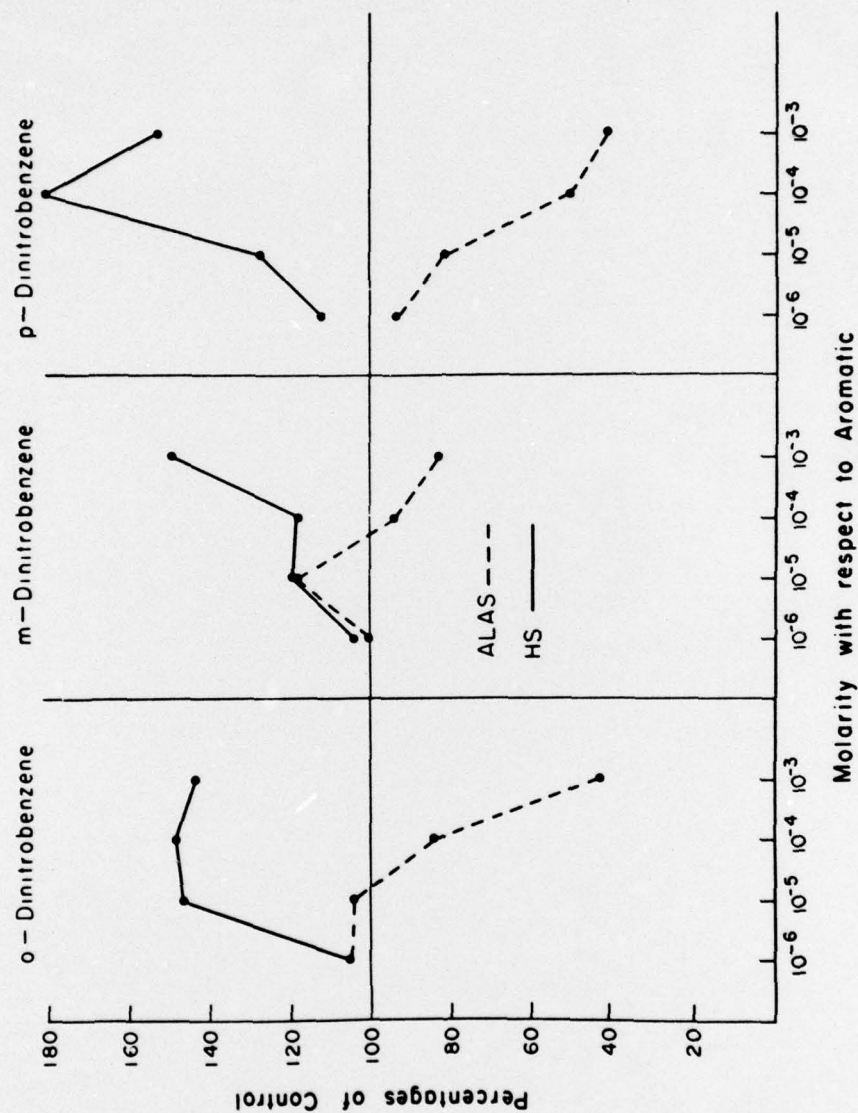


Fig. 2. The effect of the dinitrobenzenes on ALAS and HS activities. Note the inverse relationship at higher concentrations resulting in a "Y" shaped curve.

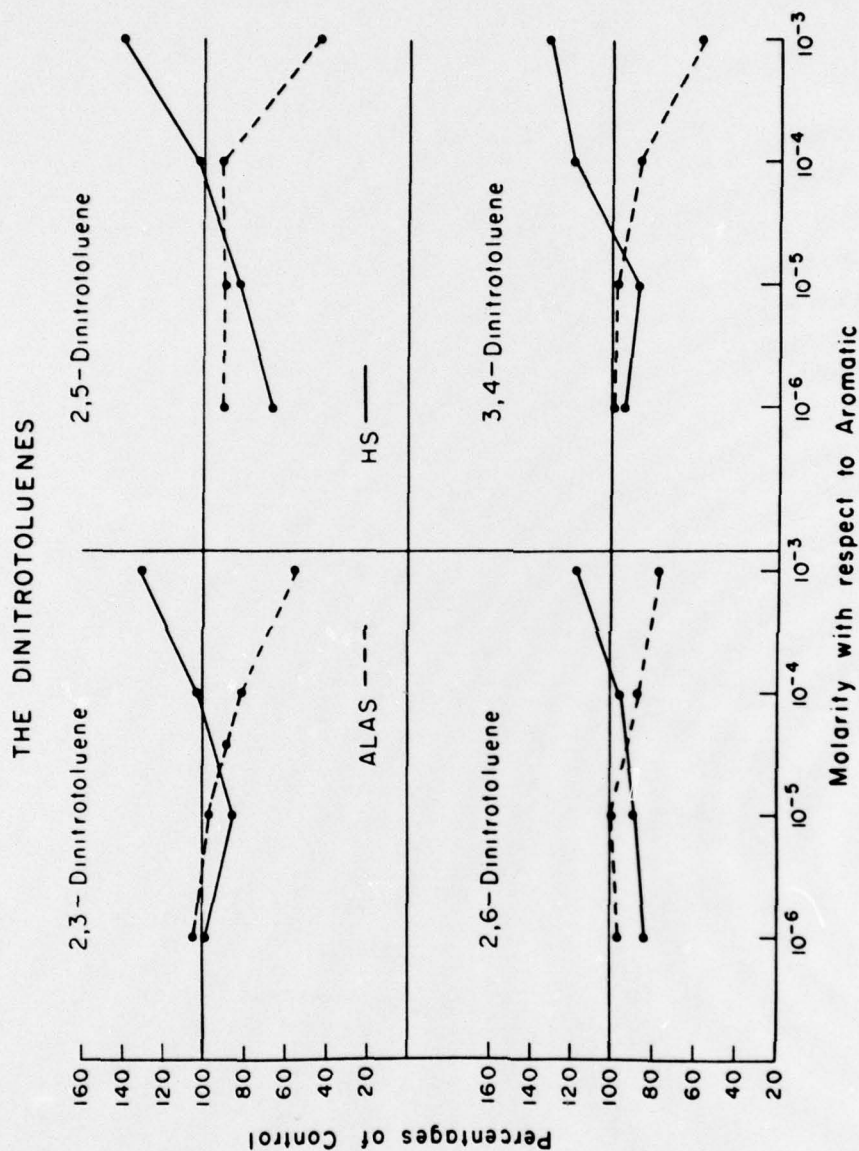


Fig. 3. A comparison of the effect of the dinitrotoluenes on ALAS and HS activities. Note the coincidence of activity in the 10^{-5} to 10^{-4} mol/l concentration range of the aromatic resulting in an "X" shaped curve.

THE TRI-SUBSTITUTED TOLUENES

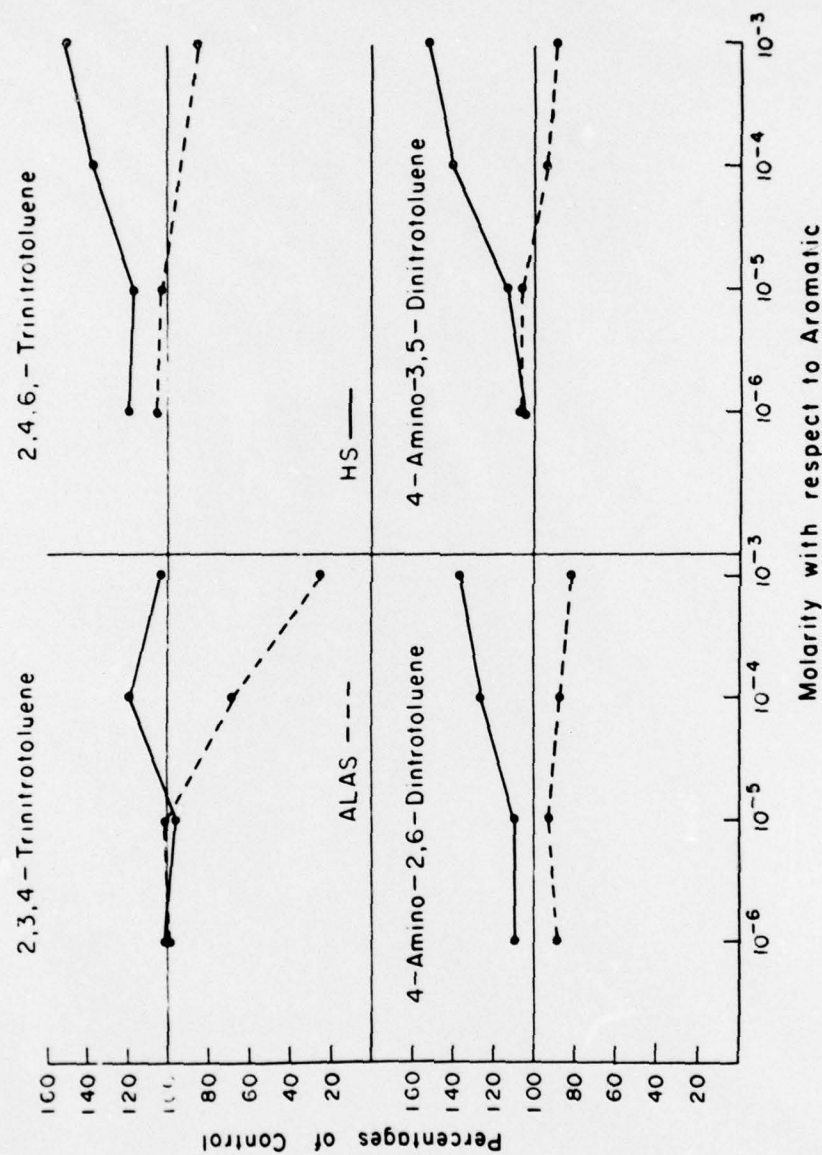


Fig. 4. A comparison of the effect of tri-substituted toluenes on the enzyme systems studied producing a "Y" shaped curve.